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Human Hair Follicle Bulge Cells are Biochemically Distinct and Possess an Epithelial Stem Cell Phenotype

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Stem cells are vital for the homeostasis of self-renewing tissues and their manipulation may have wide ranging applications, including gene therapy, wound repair, and tissue transplantation. Although rodent hair follicle stem cells have been localized to the follicle bulge, the location of human hair follicle stem cells is less clear, and their characterization has been hampered by a lack of cellular markers for the bulge area. We demonstrate that the C8/144B monoclonal antibody, originally raised against a CD8 peptide sequence, immunostains the human hair follicle bulge. We show that this antibody recognizes cytokeratin 15 (K15) in keratinocytes, and that K15-positive bulge cells possess a stem cell phenotype characterized by their slowly cycling nature, proliferation at the onset of new hair follicle growth, and high level of β_1 integrin expression. These results localize human hair follicle stem cells to the bulge and suggest that K15 is preferentially expressed in epithelial stem cells. ***Key words: cytokeratin 15/β_1 integrin. Journal of Investigative Dermatology Symposium Proceedings 4:296–301, 1999***

The homeostasis of all self-renewing tissues, including the epidermis and hair follicle, is thought to be dependent on stem cells (Wright and Alison, 1984; Morrison *et al*, 1997; Cotsarelis *et al*, 1999). As undifferentiated stem cells divide, they generate daughter cells that retain the stem cell phenotype, and daughter cells [called transit-amplifying (TA) cells] that undergo rapid proliferation and terminal differentiation to repopulate the tissue (Wright and Alison, 1984; Jones and Watt, 1993). Stem cells are generally slowly cycling, but they have a high proliferative potential and proliferate at times of tissue expansion, such as during fetal development and wound healing (Potten, 1974; Cotsarelis *et al*, 1990; Morris *et al*, 1997). On the basis of these characteristics, epithelial stem cells have been identified in the epidermis and hair follicle as keratinocytes with a high *in vitro* proliferative potential (Barrandon and Green, 1987; Kobayashi *et al*, 1993; Rochat *et al*, 1994) or as long-lived, slowly cycling "label-retaining cells" (LRC) *in vivo* (Bickenbach and Mackenzie, 1984; Cotsarelis *et al*, 1990; Morris and Potten, 1994; Lyle *et al*, 1998). Although no specific markers for epithelial stem cells are known, stem cells within the epidermis express higher levels of β_1 integrin compared with surrounding cells (Jones and Watt, 1993; Jones *et al*, 1995), and cytokeratin 19 is present, though not exclusively, in hair follicle stem cells (Lane *et al*, 1991; Michel *et al*, 1996).

Based on LRC studies in mice, we previously proposed that stem cells responsible for the cyclical regeneration of the lower hair follicle reside in an area called the hair follicle bulge (Cotsarelis *et al*, 1990). Supporting this concept, Kobayashi *et al* (1993) demonstrated that bulge cells from microdissected rat follicles have a high colony forming efficiency (CFE), an *in vitro* characteristic of stem cells; however, in *human* hair follicles, similar types of *in vitro* analyses by the same investigators and others suggested that the majority of hair follicle stem cells were located well below the level of the bulge, in the lower hair follicle outer root sheath (Rochat *et al*, 1994; Moll, 1995). In contrast, *in vitro* analyses by Yang *et al* (1993) suggested that the upper human hair follicle, which contained the bulge, possessed the cells with the highest proliferative potential (presumptive stem cells) (Yang *et al*, 1993). Therefore, the exact location of human hair follicle stem cells remained unclear. In addition, because of the lack of a marker for the bulge, investigators used the arrector pili muscle (APM) to approximate its location in dissected hair follicles; however, the AP M attachment site varies among different hair follicles and sometimes is located below the bulge (Pinkus, 1958). Thus, it has been difficult to draw conclusions about the precise location of human hair follicle stem cells, especially with respect to the bulge area.

DEFINING THE HUMAN HAIR FOLLICLE BULGE

C8/144B monoclonal antibody immunostaining delineates the human hair follicle bulge We surprisingly discovered that the C8/144B monoclonal antibody, originally raised against the carboxy-terminal peptide of the T cell protein CD8 (Mason *et al*, 1992), selectively immunostains the bulge keratinocytes of hair follicles, as well as the expected subset of lymphocytes, in tissue sections of adult human skin (Lyle *et al*, 1998) (**Fig 1**). In addition to the morphologic bulges occasionally seen in tissue sections (**Fig 1**), the C8/144B monoclonal antibody immunostains a discrete area of the outer root sheath (ORS) basal layer below the sebaceous gland duct near the attachment site of the APM of

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Abbreviations: APM, arrector pili muscle; CFC, colony-forming cell; CFE, colony forming efficiency; K15, cytokeratin 15; K19, cytokeratin 19; LRC, label-retaining cells; TA, transit-amplifying.

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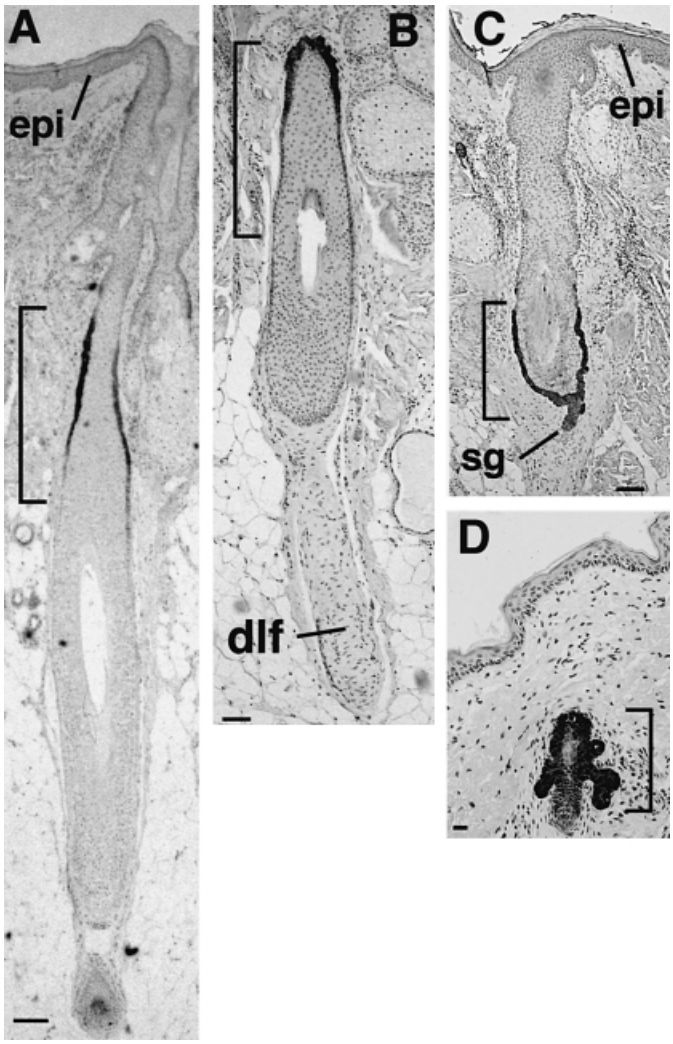

Figure 1. Immunostaining of human hair follicles. (*A*) Anagen follicle stained with the C8/144B antibody (dark grey; *scale bar*: 50 μm). The C8/144B-positive bulge cells form a cylinder of basal cells in the ORS of the lower isthmus. (*B*) Catagen follicle with C8/144B-positive bulge cells at the lower portion of the isthmus, above the degenerating lower follicle (*scale bar*: 50 μm). (*C*) Telogen follicle with C8/144B-positive bulge cells in the basal layer of the ORS surrounding the club hair as well as in the secondary germ (*scale bar*: 50 μm). (*D*) Early anagen follicle of adult skin with morphologic bulges showing positive staining (*scale bar*: 20 μm). The epidermis shows little staining. Brackets denote bulge; epi, epidermis; dlf, degenerating lower follicle; sg, secondary germ; irs, inner root sheath; ors, outer root sheath. (Reprinted with permission from *J Cell Science* 111: 3179–3188, 1998.)

anagen, catagen, and telogen follicles (**Fig 1**). The intense immunostaining extends variably above and below the exact APM attachment site, but is sharply demarcated. The upper portion of the follicle (infundibulum) and overlying epidermis are generally negative, although weaker immunostaining is occasionally evident in these areas. In approximately one-third of scalp anagen follicles in which the bulb area of the follicle was apparent, we observed an isolated cluster of positive cells in the immediate suprabulbar ORS of the lower follicle as well. The C8/144B antibody also stains the basal layer of keratinocytes at the bottom of the telogen follicle, traditionally referred to as the ''secondary germ'' or telogen germinal unit (**Fig 1**) (Headington, 1984). C8/144B-positive cells are present in the bulge area of follicles from a variety of anatomic sites such as scalp, eyebrow, and extremity. Thus, by immunostaining, the keratinocyte protein detected by C8/144B antibody appears preferentially located in the hair follicle bulge, and serves as a marker for these cells.

C8/144B monoclonal antibody recognizes cytokeratin 15 To determine the keratinocyte protein recognized by the C8/144B monoclonal antibody, we constructed and screened a human fetal skin cDNA expression library (bulges are prominent structures in fetal hair follicles (Holbrook, 1991). We screened one million clones and found 32 positive. We further screened these positive clones with Southern dot blot hybridization using ^{32}P-labeled amplified cDNA from plucked hair follicles. By nucleotide sequence homology six clones were identical to human cytokeratin 15. Thus, the C8/144B antibody likely recognizes an epitope of the K15 protein. This type of cross-reactivity of monoclonal antibodies has been previously described; Keitel *et al* (1997) and Kramer *et al* (1997) demonstrated that monoclonal antibodies commonly have cross-reactivity for homologous proteins and often demonstrate polyspecificity for unrelated proteins with completely different primary amino acid sequences. In addition, Weinberg and Yuspa (1997) reported that a monoclonal antibody raised against p53 also binds to (displays polyspecificity for) keratins.

To confirm binding of the C8/144B monoclonal antibody to K15, we immunoprecipitated the *in vitro* translated product of a full-length K15 cDNA with the antibody, and this resulted in the expected 50 kDa band (Lyle *et al*, 1998). The antibody does not recognize K15 by immunoblotting (unpublished data), suggesting that it only binds to the protein in its native conformation rather than its denatured form.

Our findings that human bulge cells selectively express K15 throughout all stages of the hair cycle in different types of follicle, indicate that the bulge is composed of a biochemically distinct, permanent population of cells within the hair follicle ORS. K15 expression in the follicle is found in the basal cell layer of the ORS of the lower isthmus and the secondary germ, the traditional site of follicular stem cells in telogen follicles (Silver and Chase, 1970). Our operational definition of the bulge, therefore, is not simply limited to the morphologic bulges that attach to the APM, but also includes these other K15-positive areas.

HUMAN HAIR FOLLICLE STEM CELLS RESIDE IN THE BULGE

Slowly cycling LRC localize to the human hair follicle bulge We determined the distribution of LRC within human hair follicles by studying human scalp grafted onto immunodeficient (CB17 Icr-*scid/scid*) mice (Lyle *et al*, 1998). Human hair follicles have been studied in this manner in the past (Gilhar *et al*, 1988). Transplanted follicles produce normal appearing hair shafts, and transit through the different phases of the hair follicle cycle (Van Neste *et al*, 1996). Similar to surgical hair transplants, hair typically sheds from grafts 2–4 wk after grafting (telogen effluvium), and then subsequently regrows. We reasoned that hair follicle stem cells must proliferate during this transition from telogen to anagen to regenerate the lower follicle that produces a new hair. To ''capture'' stem cell proliferation, we administered BrdU, a nucleoside analog, continuously for 2 wk from 3–5 wk after grafting. (Note that this technique will only identify hair follicle stem cells that are proliferating during this time period. More than likely, not *all* hair follicle stem cells proliferate at anagen onset, therefore only a subset of stem cells can be detected.) As expected, rapidly proliferating TA cells within the lower follicle and epidermis also were labeled.

To identify slowly cycling LRC, the grafts received no BrdU for 4 mo after the labeling period. During this ''chase'' period, rapidly proliferating TA cells dilute their label, and only slowly cycling LRC remain labeled. After the 4 mo chase period, LRC were present in the bulge areas of hair follicles delineated by C8/144B antibody staining (**Figs 2*A*,*B*, 3**) (Lyle *et al*, 1998). No LRC were present in anagen bulbs, or in the lower ORS of over 60 human hair follicles that were examined, thus suggesting that stem cells remain in the bulge, and do not migrate downward to the bulb during anagen onset. These findings are in line with evidence from other epithelial systems, such as the palm and corneal epithelium,

where stem cells are strongly attached to the basement membrane, and are located in permanent, well-protected areas (Lavker and Sun, 1982; Cotsarelis *et al*, 1989).

Bulge cells are quiescent throughout the hair follicle cycle, but they transiently proliferate at the onset of anagen Although others have reported that the bulge area possesses the lowest percentage of actively proliferating cells relative to other portions of the follicle (Moll, 1995; Commo and Bernard, 1997), we have established that bulge cells are slowly cycling because they retain BrdU for at least 4 mo. To further understand the proliferative behavior of the bulge cells, we examined their expression of the Ki-67 proliferation antigen (Gerdes *et al*, 1983) throughout the hair follicle cycle (Lyle *et al*, 1998). During anagen onset, many K15-positive cells express the Ki-67 proliferation antigen, indicating their entrance into the cell cycle (Lyle *et al*, 1998) (**Fig 4*A***). In later anagen, a column of proliferating Ki-67-positive, K15-negative cells forms beneath the bulge area (**Fig 4*B***). During mid-anagen, when bulge cells form a relatively flattened concentric cylinder in the ORS, the proliferating cells are predominately in the basal and suprabasal cells of the lower third of the follicle (**Fig 4*C*,*D***), as well as in the rapidly dividing matrix cells that produce the hair shaft, as has been previously reported (Moll, 1995). No mid-anagen, catagen, or telogen follicles, however, contained K15-positive bulge cells that were immunoreactive for Ki-67. Thus, bulge cells appear to be quiescent during the long anagen growth phase and briefly proliferate at anagen onset to generate rapidly proliferating progeny that regenerate a new follicle. These findings support the concept that the lower ''transient'' portion of the follicle is composed of TA cells that are important for hair growth during anagen but are dispensable for hair follicle cycling (Cotsarelis *et al*, 1990).

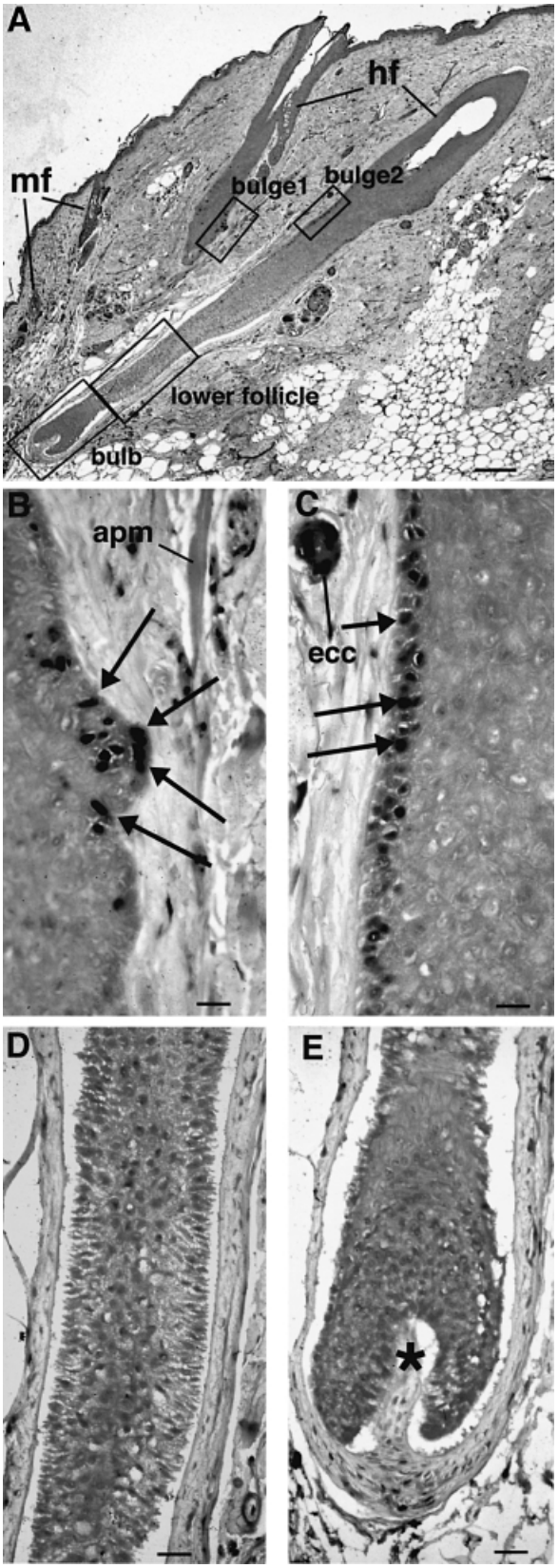

Figure 2. LRC reside in the hair follicle bulge. Human scalp grafted onto immunodeficient (*scid/scid*) mice was labeled continuously with BrdU for 2 wk and then collected after a 4 mo chase period. (*A*) Section of graft showing two follicles in human skin adjacent to mouse skin. LRC, detected by immunohistochemistry for BrdU, are present in the bulge area at the lower portion of the isthmus. (*B,C*) Represent high power view of bulges 1 and 2, respectively, and demonstrate LRC in the basal layer of the ORS at the attachment of the arrector pili muscle (*arrows*). LRC are also commonly seen in eccrine ducts. (*D,E*) Represent high power views of lower follicle and bulb, respectively. No LRC are present in lower follicle or bulb (*A*, *scale bar*: 100 μm; *B–E*, *scale bar*: 20 μm). hf, human hair follicle; mf, mouse hair follicle; apm, arrector pili muscle; ecc, eccrine duct. (Reprinted with permission from *J Cell Science* 111: 3179–3188, 1998.)

Bulge cells express high levels of β_1 integrin In addition to their slowly cycling nature, another important characteristic of stem cells is their high proliferative capacity. In keratinocytes, this proliferative capability has been studied *in vitro* by examining the clonogenicity of individual cells through serial passage. Approximately 5% of adult epidermal basal cells possess a ''holoclone'' phenotype characterized by high reproductive capacity and low level of terminal differentiation, and these cells are thought to represent stem cells (Barrandon and Green, 1987). Another indicator of proliferative capability is colony forming efficiency (CFE; colonies per number of cells plated), which is thought to correlate to the number of stem cells in a tissue (Jones and Watt, 1993; Kobayashi *et al*, 1993). Epidermal keratinocytes selected for high levels of β_1 integrin expression (integrin bright) demonstrate a higher CFE than epidermal keratinocytes with low integrin expression (integrin dull) (Jones *et al*, 1995). Integrin bright epidermal keratinocytes possess fewer S-phase cells on average than integrin dull cells, suggesting that these cells divide infrequently under steady state conditions. Although integrin bright cells likely encompass both stem cells and TA cells (Jones *et al*, 1995; Li *et al*, 1998), high surface β_1 integrin expression is a characteristic of epidermal stem cells, and integrin bright cells localize to specific areas of the epidermis, such as the tips of the rete ridges in glabrous skin, where stem cells were described in monkeys (Lavker and Sun, 1982). Moll (1995) also found intense β_1 integrin immunostaining of the basal cells of the bulge area and of the bulb matrix

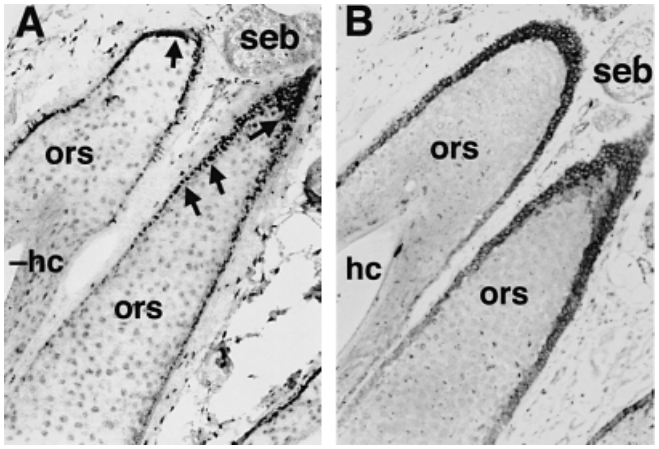

Figure 3. C8/144B-positive LRC. Human skin/SCID mouse chimera labeled with BrdU. After a 4 mo chase period, serial sections were immunohistochemically stained with anti-BrdU (*A*) and C8/144B (*B*) monoclonal antibodies. LRC (dark black nuclear stain in *A*) are present in C8/144B-positive bulge cells (dark grey cytoplasmic stain in *B*) in the basal layer of the ORS. *Scale bar*: 20 μm. hc, hair canal; ors, outer root sheath; seb, sebaceous gland

keratinocytes, although quantitative analysis of integrin levels using confocal microscopy was not done.

To accurately localize integrin bright keratinocytes within the hair follicle, and analyse their relationship to K15-positive bulge cells, we examined immunofluorescently stained hair follicle whole mounts using confocal microscopy. Integrin bright cells co-localize precisely with K15-positive cells in the bulge area of anagen follicles (Lyle *et al*, 1998) (**Fig 5**). ORS cells above and below the K15-positive bulge area show only weak β_1 integrin positivity. The K15-positive cells express approximately 4-fold higher levels of β_1 integrins than neighboring ORS cells (**Table I**) The K15-negative hair matrix cells express an intermediate level of β_1 integrin with a ratio of approximately 2.5:1 between K15-positive bulge cells and the more differentiated matrix cells. In telogen follicles, K15 expression co-localizes with intense β_1 integrin staining in the basal layer of the ORS surrounding the club hair, whereas the K15-negative suprabasal cells have a similar intermediate β_1 integrin positivity as in the matrix cells (**Fig 5*C*,*D***).

Serial sections of frozen scalp skin, stained individually for K15 or β_1 integrin, also demonstrate that K15-positive bulge is qualitatively ''integrin bright''. In addition, the K15-positive cells present in the lower portion of some follicles show intense β_1 integrin immunofluorescence. Because β_1 integrins mediate the adhesion of freshly isolated keratinocytes to substrates in culture (Adams and Watt, 1991), the high level of integrins in the bulge area may explain why follicles in explant culture develop outgrowths more frequently from this area of the follicle (Yang *et al*, 1993; Moll, 1996). Furthermore, because integrins are known to link keratin filaments with other cytoskeletal and plasma membrane proteins (Fuchs and Cleveland, 1998), the striking co-localization of K15 and intense β_1 integrin expression within bulge cells suggests that these two proteins may interact, and that their expression may be co-regulated. Future studies to address this issue may lead to a better understanding of the function of these proteins in maintaining the epithelial stem cell phenotype.

CYTOKERATINS AS MARKERS OF DIFFERENTIATION WITHIN THE HAIR FOLLICLE

Within stratified squamous epithelia, such as the epidermis, hair follicle, and cornea, cytokeratin expression is generally restricted to well-defined populations of cells in similar states of differentiation (Schermer *et al*, 1986; Schirren *et al*, 1997; Lane *et al*, 1999). For example, within the epidermis, the keratin pair 5/14 is expressed predominantly in the basal layer of the epidermis, which is

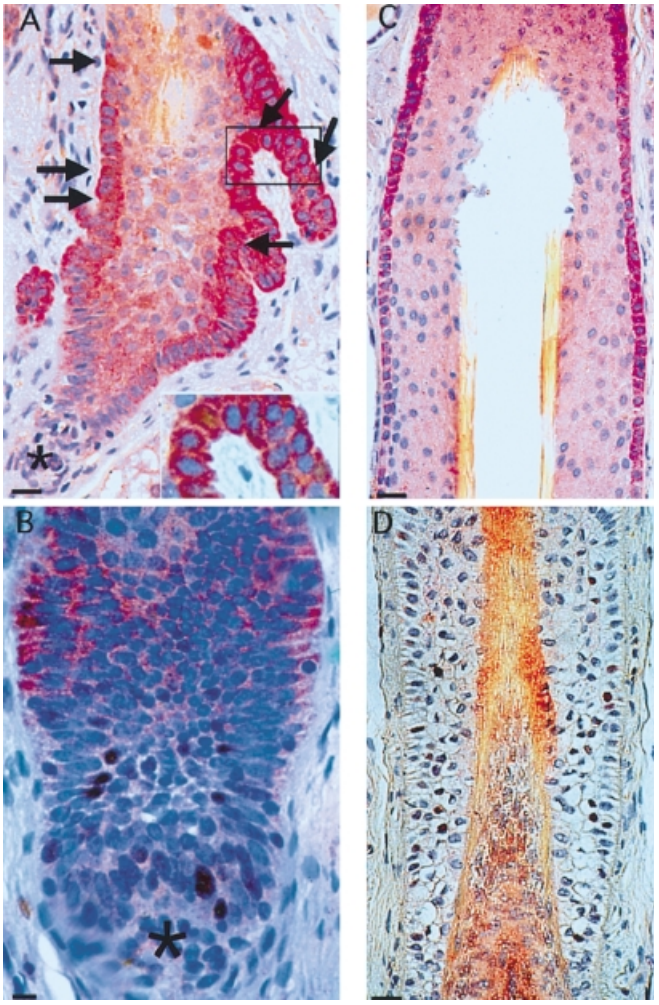

Figure 4. Human scalp double-stained with anti-Ki-67 (brown/black nuclear staining) and C8/144B (red cytokeratin staining) antibodies. (*A*) Follicle at anagen onset showing C8/144B-positive bulge cells co-expressing Ki-67 proliferation antigen (*arrows* indicate double-labeled cells; *scale bar*: 25 μm). (*B*) Early anagen follicle showing C8/144B-positive, Ki-67-negative bulge and a downgrowth of C8/144B-negative, Ki-67-positive cells above dermal papilla (* dermal papilla, *scale bar*: 10 μm). Mid-anagen follicle has no proliferating cells in C8/144B-positive bulge area (*C*) and numerous proliferating cells in suprabasal and basal layers of the lower portion of follicle (*D*). *Scale bars*: 25 μm. (Reprinted with permission from *J Cell Science* 111: 3179–3188, 1998.)

composed of proliferating, relatively undifferentiated keratinocytes (Coulombe *et al*, 1989). As keratinocytes leave the basal layer and become postmitotic and more differentiated, they cease production of K5/14, and begin to produce K1/K10. Similarly, in the cornea, the more differentiated central corneal epithelium expresses K3/K12 throughout the basal and suprabasal layers, whereas peripheral cornea (limbus), which contains stem cells (Cotsarelis *et al*, 1989), expresses these keratins only in the more differentiated suprabasal cells (Schermer *et al*, 1986).

Because cytokeratin 19 (K19) is thought to be a stem cell marker (Michel, 1996), we examined K19 expression in whole-mounted human follicles and frozen tissue sections using immunofluorescent and immunohistochemistry. Although K19-positive cells co-localized with K15-positive cells in the basal layer of the ORS within the bulge area, K19-positive cells extended downward throughout the entire basal layer of the ORS from the bulge to the bulb in whole-mounted follicles (Lyle *et al*, 1998). K15-positive cells, therefore, comprise a subset of K19-positive cells in the human hair follicle. These data suggest that both K15 and K19 are markers of relatively undifferentiated keratinocytes in the bulge;

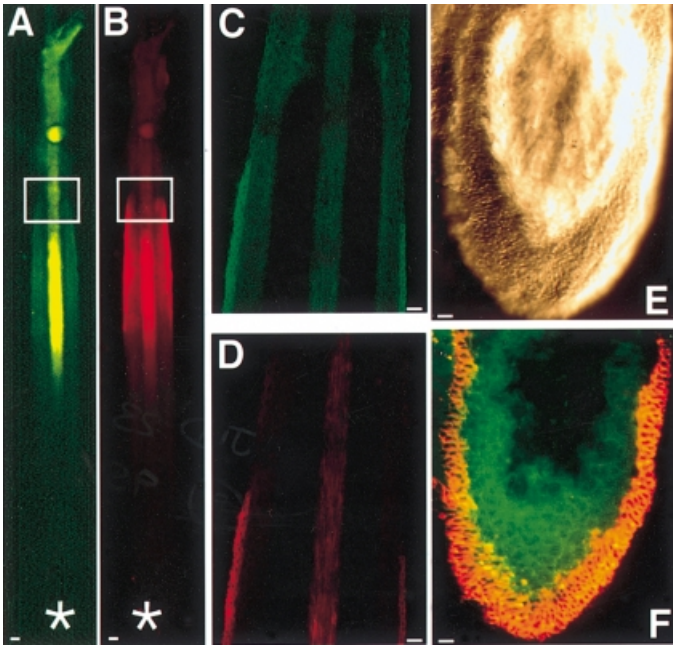

Figure 5. Co-localization of C8/144B staining and β_1 integrin expression in whole-mounted follicles examined by confocal microscopy. Bulge area of anagen follicle shows intense β_1 integrin expression (green, *A*, *C*)and C8/144B staining (red, *B*, *D*). *Scale bars*: 25 μm. ORS cells above the bulge area show very weak β_1 integrin expression. In telogen follicles (*E*-phase contrast), C8/144B and intense β_1 integrin immunofluorescence co-localize to the basal layer of the ORS surrounding the club hair (*F*). *Scale bars*: 10 μm. The double exposure of green β_1 integrin and red K15 immunofluorescence produces organ in double-labeled cells (*F*). The K15-negative suprabasal cells surrounding the club hair possess an intermediate level of β_1 integrin expression. * Dermal papilla. (Reprinted with permission from *J Cell Science* 111: 3179–3188, 1998.)

Table I. β_1 Integrin expression of hair follicle epithelium

	Bulge cells	Nonbulge ORS	Matrix cells
Fluorescence[a]	161+/–6	44+/–2	66+/–2
Ratio[b]		3.7:1	2.4:1

[a]Average fluorescence measured in arbitrary units of pixel intensity on a linear scale from 0 to 255 (n=25).
[b]Ratio of fluorescence intensity (integrin bright bulge cells:integrin dull cells).

however, K15 appears restricted to the permanent portion of the follicle containing β_1 integrin bright cells and LRC, whereas K19 is present in more differentiated TA cells in the lower follicle as well. These findings agree with those of Jones *et al* (1995), who suggested that only a subset of follicular K19-positive cells represent true stem cells. Based on our data, it is likely that the K15-positive keratinocytes comprise this subset. Our studies also support the notion that loss of K15 expression may be one of the earliest signs of the transition from stem to TA cells, and that the K15-negative/K19-positive phenotype may indicate that these cells are ''early'' TA (TA_1) cells.

Interestingly, we also identified K15 cells clustered in the lower ORS of approximately one-third of anagen follicles (Lyle *et al*, 1998), in an area where Rochat found 24% of the total CFC present within dissected adult follicles (Rochat *et al*, 1994). This area corresponds to the fragment that demonstrated the highest CFE and a significant proportion of CFC in plucked as well as dissected follicles (Moll, 1995). Because these few discrete cells were also qualitatively integrin bright, they may represent very early TA cells or stem cells that have migrated down during anagen. Although the significance of this biochemical heterogeneity of the lower ORS is unclear, perhaps the presence or absence of these K15-positive cells in the lower ORS indicates the position of the follicle within the hair follicle cycle (e.g., early *versus* late anagen).

Recently, two publications have appeared that examined K15 expression in human (Waseem *et al*, 1999) and sheep skin (Whitbread and Powell, 1999). Waseem *et al* (1999) reported the presence of K15, by immunostaining, in the epidermis, and that K15 staining was increased in skin from epidermolysis bullosa simplex patients. They also noted that K15 immunostaining was decreased in hyperproliferative epidermis (psoriasis and overlying hypertrophic scars), and suggested that K15 may be expressed ''predominantly in stem cell populations of the basal keratinocytes''. These authors found K15 in the hair follicle ORS, not in the hair bulb. Further studies are needed to better define the expression of K15 in the epidermis, and its relationship to epidermal stem cells.

Whitbread and Powell (1998) examined K15 mRNA expression by *in situ* hybridization in sheep. Their results differ significantly from our findings in human skin. For example, they found K15 expression in the basal layer throughout the hair follicle ORS and hair bulb matrix, although they describe an absence of signal in the isthmus, at or near the bulge in ''over half of large, medullated hair follicles''. These findings may reflect differences in the duration of anagen among different species. In particular, sheep hair follicles are thought to stay in anagen throughout the entire lifetime of the animal. Thus the organization of stem cells and K15 within the sheep follicle may be substantially different than human and mouse follicles, which eventually enter catagen. Whitbread and Powell (1998) attempted to address this issue by generating transgenic mice expressing the entire sheep K15 gene. This construct included the regulatory regions (e.g., sheep K15 promoter/enhancer) controlling expression of K15 within the sheep. Therefore, it perhaps is not surprising that K15 expression in these transgenic mouse hair follicles was similar to its distribution in the sheep, although the K15-negative cells in the isthmus were not observed in the mouse. Further studies on the hair cycle dependent expression of K15 in normal mice are needed.

In mice, both K15 and K14 can pair with K5 in the skin and other stratified epithelia (Lloyd *et al*, 1995). In neonatal mice, both K15 and K14 are limited to the basal layers of epidermis, ORS of hair follicles, and cornea, as well as internal stratified epithelia such as tongue and forestomach. K15 appears to be a minor component in the skin of neonatal mice, which shows a high K14/K15 ratio, whereas K15 demonstrates a higher expression than K14 in esophagus of neonatal mice. The high ratio of K15/K14 of neonatal mouse esophagus dramatically reverse in older mice as K14 becomes the predominant keratin. This normal postnatal, developmental switch to K15-predominant intermediate filaments may be responsible for the brittleness and blistering observed in adult but not neonatal K14 (–/–) knockout mice (Lloyd *et al*, 1995). Lloyd hypothesized that K5-K14 filaments may have greater structural integrity, required of adult mice eating solid food, that K5-K15 filaments prevalent in the esophagus of neonatal mice with a liquid diet. Similar types of alterations in K15 expression may account for age-related changes in the physical properties of skin.

CONCLUSIONS AND IMPLICATIONS

Within murine skin, hair follicle stem cells are generally slowly cycling, but they proliferate at the onset of anagen or in response to population depletion caused by wounding or other proliferative stimuli (Silver *et al*, 1967; Wilson *et al*, 1994). The *in vivo* proliferative behavior of human hair follicle bulge cells during the hair cycle previously has been hindered by the lack of a marker for these cells. Using the C8/144B antibody to define the bulge, we have shown that the bulge is composed of K15-positive, integrin bright keratinocytes that comprise a subset of K19-positive ORS cells (Lyle *et al*, 1998). Our results parallel our earlier findings in mice (Cotsarelis *et al*, 1990; Wilson *et al*, 1994), and clearly show

that bulge cells are extremely slowly cycling – retaining BrdU for over 4 mo – but proliferate at the onset of anagen to produce a new lower follicle. An analysis of proliferative activity using the combination of K15 and Ki-67 immunostaining could potentially serve as a bioassay to study the effectiveness of an agent's ability to stimulate follicular stem cells.

A better understanding of stem cells in the bulge should impact the field of gene therapy. Because of their slowly cycling nature, bulge cells may be ideal targets for gene therapy, and their transduction is a prerequisite for overcoming current difficulties in achieving long-term expression of transgenes in the rapidly proliferating epidermis and hair follicle (for review, see Khavari, 1998). Theoretically, transgenes integrated into the bulge cell genome will result in expression of these genes not only in bulge cells, but in their progeny (TA cells) as well. With the recent advent of methodologies resulting in replacement of mutant genes with wild-type sequences through homologous recombination (Alexeev and Yoon, 1998), permanent genotypic alterations in the epithelial portion of the hair follicle and possibly epidermis could be achieved by targeting bulge cells. The accessibility of the bulge cells using topical liposome delivery of foreign DNA seems feasible based on the work of Li and Hoffman (1995), showing successful transduction of hair follicle keratinocytes. Consideration of hair follicle cycling and bulge cell proliferation (identified, for example, as K15-positive/Ki67-positive cells) will be critical to the success of gene therapy attempts, as expression and integration of DNA is generally dependent on proliferation of host cells.

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